Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol

Supplemental Assay Method for Detection of Extraneous Bacteria and Fungi in Live Bacterial Vaccines

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1. Introduction

1.1 Background

This Supplemental Assay Method (SAM) describes the test procedure used to detect viable extraneous bacteria and fungi in live bacterial vaccines, per the Code of Federal Regulations, Title 9 (9 CFR) Part 113.27(b). If extraneous viable bacteria or fungi are present, they will be detected by morphology, gram stains, differential agar, and agar slants when compared to appropriate positive control.

1.2 Keywords

Live bacterial vaccines, purity test, extraneous bacteria, extraneous fungi

2. Materials

2.1 Equipment/instrumentation

2.1.1 Sterile syringes--size as needed for rehydration and inoculation (vacutainer needles may be used in some cases)

2.1.2 Sterile

2.1.3 Disposable inoculating loops

2.1.4 Microscope slides

2.1.5 Bunsen burner

2.1.6 Biosafety cabinet

2.1.7 30°-35°C incubator

2.1.8 20°-25°C incubator

2.2 Reagents/supplies

2.2.1 Media: Fluid Thioglycollate medium (FTM) **Section 8.2,** Trypticase Soy broth (TSB) **Section 8.1,** Triple Sugar Iron Agar (TSIA) **Section 8.5**, MacConkey agar (MC) **Section 8.4**, Trypticase Soy agar (TSA) **Section 8.3**

2.2.2 70% ethyl alcohol

2.2.3 Sterile purified water--volumes vary

2.2.4 Sterile clothes; coveralls, gloves, head cover, mask, sleeves

2.2.5 Protective eyewear

2.2.6 4 x 4 in sterile gauze pads

3. Preparation for the test

3.1 Personnel qualifications/training

3.1.1 The personnel performing the test must have experience or training in this protocol. This includes knowledge of aseptic biological laboratory techniques and preparation, proper handling, and disposal of biological agents, reagents, tissue culture samples, and chemicals. The personnel must also have knowledge of safe operating procedures and policies and Quality Assurance (QA) guidelines of the Center for Veterinary Biologics-Laboratory (CVB-L) or equivalent, as well as training in the operation of the necessary laboratory equipment listed in **Section 2.1.**

3.2 Preparation of equipment/instrumentation

3.2.1 Turn the biosafety cabinet on 1 hr prior to testing.

3.2.2 Monitor incubators daily according to the current version of GDOCSOP0001.

3.2.3 Monitor freezers and coolers used for the storage of biologics for temperature daily, according to the current version of GDOCSOP0003.

3.3 Preparation of reagents/control procedures

3.3.1 Positive control organisms must be the equivalent of the live bacterial product that is to be tested. These organisms determine the growth promoting qualities of the medium and provides a comparison for the organism in the product.

3.3.2 Conduct growth promotion tests on the batch of TSB and FTM used in this test according to the current version of STSAM0900.

3.3.3 Technique (tech) Controls: Inoculate 20 test vessels of media using sterile water prepared by the National Veterinary Services Laboratories (NVSL) media preparation department. Use the same lot of vials as used in rehydration of the biological product or products. If the products had their own sterile diluent, use 10 serum vials containing 5 ml water each. Inoculate 0.2 ml water into 20 test vessels and incubate at each temperature as the original test.

3.3.4 Negative or Media Controls: Incubate 20 representative test vessels of uninoculated medium to confirm sterility. These vessels are incubated at each temperature for the duration of the original test.

3.4 Preparation of the samples

3.4.1 Samples to be tested are received from the Biological Material Processing Section (BMPS) according to the current version of STSOP0001.

3.4.2 Check in biological samples by comparing the sample codes and serial numbers on the vial and to the accompanying computer printout sheet and the appropriate logbook, according to the current version of STSOP0010.

3.4.3 Order sterile water for the diluent if none accompanies the product or if there is none on hand. Order 10 extra for the controls.

4. Performance of the test

4.1 Disinfect the samples.

4.2 Number media test vessels to coincide with the serials to be tested.

4.3 Disinfect the tops of the samples and flame by using a Bunsen burner.

4.4 Rehydrate each vial, if needed, using a syringe or a vacutainer needle with accompanying sterile diluent or sterile water.

4.5 Inoculate 10 test vessels containing TSB and 10 of FTM with 0.2 ml from each 10 vials of product.

4.6 Inoculate 2 tubes of each temperature using positive control which corresponds to the product being tested.

4.7 Inoculate tech controls using 0.2 ml of sterile water.

4.8 Place test vessels containing the FTM into an incubator at 30°-35°C. Place test vessels containing the TSB into an incubator at 20°-25°C. Incubate negative and tech controls in the same way as the product.

4.9 Inoculate growth promotion tests on each media type according to the current version of STSAM0900.

4.10 If after 14 days of incubation all 10 tubes look the same, sub culture the first 3 tubes of each temperature. Media used in sub culturing are McConkey agar, TSA, and TSIA slant. Make a slide of the culture and gram stain. Sub culture 1 tube of the positive control at each temperature to compare. If any of the tubes at either temperature look macroscopically different as opposed to all other tubes, sub culture those tubes in order to rule out contamination.

4.11 Incubate sub cultures for 3 days at each temperature. Compare colony morphology on the agars and compare gram stain morphology on the slides to those of the positive control. If the colony morphology is different than that of the positive control, make a gram stain of the different colonies and compare to the slides of the positive control.

5. Interpretation of the test results

5.1 Products and positive controls that have no growth are considered satisfactory after the end of the 14 days ncubation. Compare sub cultured products to the controls. Compare colony morphology, gram stains, and growth in broth. If product growth matches that of the control test vessels, and gram stains in at least 9 of the 10 test vessels at each temperature, the serial is satisfactory, (SAT).

5.2 If product and control do not agree in 2 or 3 test vessels the product will be retested (RT). Order 20 new vials of the product and a delayed test report. Carry out the RT in the same manner, **(Section 4.1-4.11)** using the 20 new vials. If the retest is not conducted within 21 days, the product is UNSAT by the results of the first test.

5.3 If extraneous growth occurs in 4 or more vessels and is not indicative of the positive control, the test is considered unsatisfactory (UNSAT).

5.4 If extraneous growth occurs in 2 or more of the test vessels at either temperature of incubation, of the retest, the serial is unsatisfactory.

5.5 Enter a no test (NT) if the positive control did not grow or growth was different than expected. A NT is also entered when the technique or the negative controls are contaminated.

6. Report of test

6.1 Record test results in the testing log book (STFRM027P) and on the computer test sheet for each serial tested. Initial and date on the test sheet.

6.2 Enter test results into CVB computer as per current version of STSOP0021. A printout of each serial tested will be received. Compare the printed sheets to the log book for accuracy.

6.3 Forward the test printout and the log book to the supervisor or microbiologist to review and sign.

6.4 Validate the tests results using the current version of STSOP0021.

6.5 File the signed and validated worksheets in Cytology/Sterility (CS) files under the first 2 numbers of the serial's product code. File the computer sheet from BMPS by test code in the same file drawer.

7. References

7.1 Code of Federal Regulations, Title 9, Parts 113.27b U.S. Government Printing Office, Washington, D.C. 1996.

7.2 The U.S. Pharmacopoeia, 1985, Vol. 21, pp 1151-1160, Mack Publishing Co., Eaton, Pa.

8. **Appendices**--media formulations

8.1 NVSL Media Formulation #10423

Trypticase Soy Broth (TSB)

Trypticase Soy Broth	30 g
QH_2O	1000 ml

Autoclave 20 min at 121°C

8.2 NVSL Media Formulation #10135

Fluid Thioglycollate Medium (BBL)

Fluid Thioglycollate medium	29.5 g
QH_2O	1000 ml

Mix and heat to boiling.
Autoclave 20 min at 121°C.

8.3 NVSL Media Formulation #10487

Trypticase Soy Agar (TSA)

Trypticase Soy Agar (BBL)	40 g
QH_2O	1000 ml

Autoclave 20 min at 121°C.

8.4 NVSL Media Formulation #10217

MacConkey Agar

MacConkey Agar (BBL)	50 g
QH_2O	1000 ml

Autoclave 20 min at 121°C.

8.5 NVSL Media Formulation #10406

Triple Sugar Iron Agar

Triple Sugar Iron Agar Slants (TSIA)	65 g
QH_2O	1000 ml

Autoclave 15 min at 121°C.
Slant tubes at a 20° angle.